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Short sequence-paper

cDNA cloning and expression of a class II acidic chitinase from sweet orange [1,2]

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Abstract

A citrus cDNA encoding a class II acidic chitinase was isolated from a nonembryogenic cell line of sweet orange using the tobacco cDNA clone PROB3. Northern blot analysis revealed that the corresponding mRNA is expressed in young, green bark but not in leaves, roots, or flavedo.

Keywords: Hydrolase; Tissue culture; Pathogenesis-related protein; (Citrus); (Plant)

Although plants lack chitin they produce chitinases. Chitinases are among the enzymes identified as pathogenesis-related (PR) proteins that can be induced by a variety of chemical elicitors or pathogen infection [1,2] and are believed to be involved in a plant's defense response [3]. A defensive response to fungal infection has been demonstrated for some chitinases alone [4] or in combination with plant glucanases [5]. A promoter element has been identified in a tobacco chitinase gene that is responsive to a fungal elicitor [6]. Lin et al. [7] transformed rice plants with a rice-derived chitinase gene under the control of the cauliflower mosaic virus 35S promoter. The transformed plants showed varying levels of resistance to *Rhizoctonia solani*-induced sheath blight that correlated with the level of chitinase gene expression.

We have reported on the identification and characterization of acidic and basic hydrolases from nonembryogenic sweet orange callus that have chitinase or chitinase/chitosanase activities [8–10]. The N-terminal sequence of these hydrolases indicate that some isoforms with chitinase and chitosanase activities belong to class III chitinases, while an acidic isoform with only chitinase activity belongs to class II chitinases. Recently, we have shown that larvae of the sugarcane rootstalk borer weevil, *Diaprepes abbreviatus*, can induce several chitinases in the roots of a variety of citrus rootstocks by feeding activity [11], a response that categorizes these chitinases as PR-proteins [12].

In this study we report the identification, cloning, sequencing, and expression of a gene for a class II chitinase isolated from a sweet orange callus cell line.

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[1] The nucleotide sequence reported has been entered into the EMBL Data Library under the accession No. Z70032.

[2] Mention of a trademark, warranty, propriety product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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Single stem seedlings of juvenile (<2 years) sweet orange (*Citrus sinensis* L. Osb. cv. Valencia) were grown in a 50% shaded greenhouse under natural-day conditions. Leaf, root, and bark samples were obtained from these plants. Leaf and flavedo tissues were obtained from mature field-grown 'Valencia' trees (>5 years). A 'Valencia' nonembryogenic cell line, Val88-1, was initiated and maintained as previously described [13].

Nucleic acid isolations from citrus tissues and cell cultures were done as previously reported for genomic DNA [14], total RNA [15] and poly-adenylated RNA [16]. Poly-adenylated mRNA were isolated from plant tissues and cell cultures using 100 mg and 250 mg of tissue, respectively. Each sample was purified with 0.75 mg of BioMag Oligo $(dT)_{20}$ (Perceptive Diagnostics, Cambridge, MA) according to the manufacturer's protocol. Samples were eluted in 35 μl of deionized water and reduced to 7 μl in a DNA Speedvac (Savant Instruments, Farmingdale, NY).

A cDNA library was constructed using poly-adenylated mRNA isolated from non-embryogenic 'Valencia' orange callus tissue (Val88-1). Val88-1 is a friable, nonembryogenic cell line that has been in culture for 7 years and from which organogenesis has not been observed. It produces acidic and basic chitinase/chitosanase isoforms [8–11]. The cDNA was synthesized, ligated into the lambda ZAP-XR vector (Stratagene, La Jolla, CA), and packaged according to the manufacturer's protocol. The library was amplified once and the resulting library stock was used for screening. Aliquots of the amplified library were screened on 150 mm plates at a density of 35 000 pfu/plate as described by Sambrook et al. [17]. Biotin labelled insert DNA from the tobacco chitinase cDNA clone Prob 3 [18] was used as a probe. Individual clones identified in the library were plaque-purified and subcloned using the manufacturer's in vivo excision protocol (Stratagene, La Jolla, CA).

DNA sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL). Nucleotide sequences and derived protein sequences were analyzed using the PC/GENE software package (Intelligenetics Inc., Mountain View, CA).

Poly-adenylated mRNAs isolated from leaves, fruit flavedo, roots, bark, and cell line samples were separated (1 or 2 μg/lane) by glyoxal/dimethyl sulfoxide agarose gel electrophoresis [17]. RNAs were transferred to Immobilon-S nylon membrane (Millipore Corp., Bedford, MA) using $20\times$ SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7) as the transfer buffer. A biotin-labeled oligo(dT) 30-mer probe (R and D Systems, Minneapolis, MN, USA) was used to verify the presence and transfer of mRNA onto the hybridization membrane. Labelling of the cDNA insert, hybridizations, and chemiluminescent detection were performed using the random primer biotin labeling NEBlot Phototop system (New England BioLabs, Beverly, MA, USA).

The isolated cDNA clone (pACVC-1) was used to evaluate gene expression in Val88-1, leaves, bark, roots, and fruit flavedo from juvenile and mature sweet orange plants. Val88-1 is callus derived from juice vesicles and does not regenerate shoots via embryogenesis or organogenesis.

Approximately 100 000 recombinants from the Val88-1 cDNA library were screened using a biotin labelled tobacco cDNA clone PROB 3 [18]. Eleven positive clones were identified containing inserts from 900 bp to 1100 bp. The 5′ ends of the 11 clones were partially sequenced. The sequences obtained were identical or nearly identical and differed primarily in the length of the 5′ region of the cDNA inserts. The largest clone pACVC-1 (1079 bp) was completely sequenced (Fig. 1) and contains an 876 bp open reading frame starting at nucleotide 20. The sequence obtained from pACVC-1 is homologous to other plant class I and II chitinase genes as inferred from sequence similarities.

The 292 amino acid pACVC-1-encoded polypeptide (ACVC-1) is homologous to tobacco class I and II chitinases as inferred from sequence alignments. Because ACVC-1 has $>50\%$ sequence similarity to tobacco class I chitinase, but lacks an N-terminal cysteine-rich lectin-binding domain it is categorized as a class II chitinase [19]. However, pairwise similarity scores [20] with ACVC-1 were higher for class I chitinases (141–149) than the class II chitinases (118–119; Table 1). ACVC-1 also has a 7 amino acid C-terminal region similar to that found in many tobacco class I chitinases, but which is not present in the tobacco class II chitinases (Table 1). ACVC-1 has 2 chitinase class I signature sequences as defined by PROSITE [21]. The first is a 23 amino acid sequence

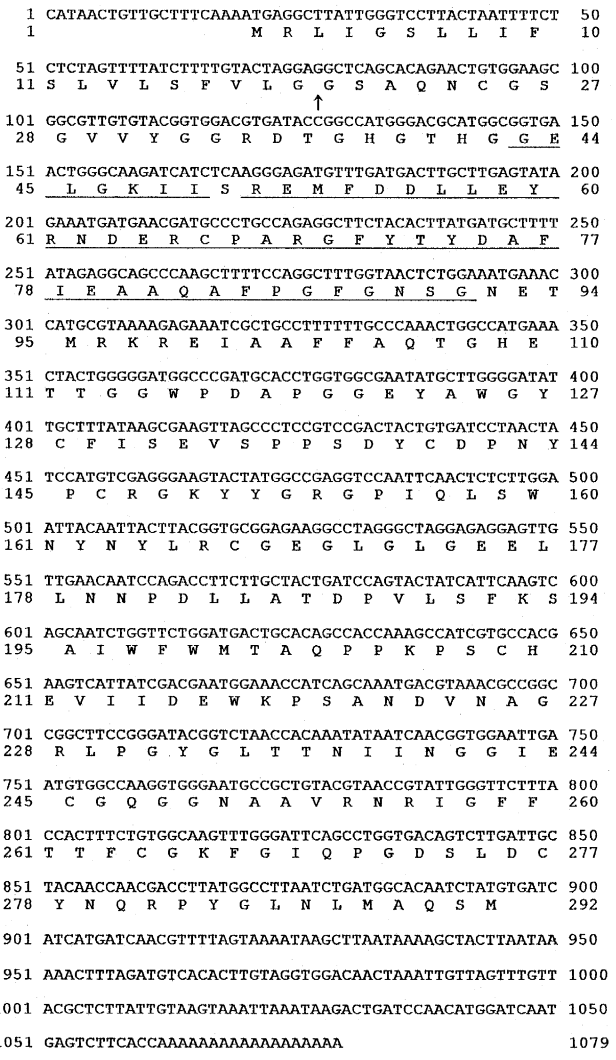

Fig. 1. Nucleotide sequence and derived amino acid sequence of pACVC-1. A 48 amino acid region homologous to the N-terminal sequence of A4-AN1 [9]. The ↑ represents a cleavage site of a possible N-terminal secretory sequence.

from 66 to 88 (Fig. 1) and the second is an 11 amino acid sequence from 190 to 200.

The 48 amino acid region of ACVC-1 starting 43 amino acids from the N-terminal methionine matches, with one exception, the N-terminal sequence of A4-AN1 (Fig. 1), an acidic chitinase isolated from Val88-1 [9]. The eighth amino acid serine differs from the phenylalanine reported for A4-AN1. Southern analysis of genomic DNA was performed using the entire pACVC-1 cDNA (1079 bp). Based on the number of hybridizing fragments (Fig. 2), pACVC-1 may be a member of a small gene family. The encoded polypeptide contains a hydrophobic N-terminal signal peptide with a potential cleavage site between amino acids 19 and 20 (Fig. 1) that conforms to the $(-3,-1)$ rule [22]. The isoelectric point of ACVC-1 is estimated to be 4.82 for the entire coding sequence (aa's 1–292) and 4.52 for the mature polypeptide (aa's 43–292). The p*I* for the A4-AN1 chitinase is 4.56 [9].

Poly-adenylated mRNA was isolated from leaves, green bark, and roots of 'Valencia' seedlings, and leaves and green flavedo of mature 'Valencia' trees and fruit. Northern blots of these mRNAs were probed at high stringency using the pACVC-1 insert. mRNAs hybridizing to the pACVC-1 probe were detected in young green bark and Val88-1 nonembryogenic callus but were not detected in the other tissues (Fig. 3). All Northern blots were stripped of pACVC-1 probe and reprobed with a biotin-labeled oligo(dT) 30-mer probe to verify the transfer of mRNA onto the hybridization membrane [32]. The resulting black smears verified the transfer of the mRNA. A series of tubulin and actin probes were tested as positive controls. None could be used as they were either not expressed or not expressed equally in all tissues (unpublished data).

Table 1
Pairwise similarity scores [20] and C-terminal region of pACVC-1 encoded polypeptide and 5 tobacco chitinases

	1	2	3	4	5	6
1	xxxx	141	148	149	118	119
2	141	xxxx	260	263	133	137
3	148	260	xxxx	314	138	140
4	149	263	314	xxxx	138	140
5	118	133	138	138	xxxx	236
6	119	137	140	140	236	xxxx
1	S L D C Y N Q R P Y G L **N L M A Q S M**					
2	N I D C G N Q K S F N S **G L L L E T M**					
3	N L D C G N Q R S F G N **G J J V D T M**					
4	N L D C G N Q R S F G N **G L L V D T M**					
5	N L D C Y N Q R N F A Q G					
6	N L D C Y N Q R N F G Q G					

1 = pACVC-1 encoded polypeptide; 2 = endochitinase 3 precursor [30]; 3 = endochitinase B precursor [31]; 4 = endochitinase A precursor [23]; 5 = acidic endochitinase P precursor [2]; 6 = acidic endochitinase Q precursor [2]; C-terminal region of pACVC-1 encoded polypeptide (1), Class I chitinases (2,3,4), class II chitinases (5,6).

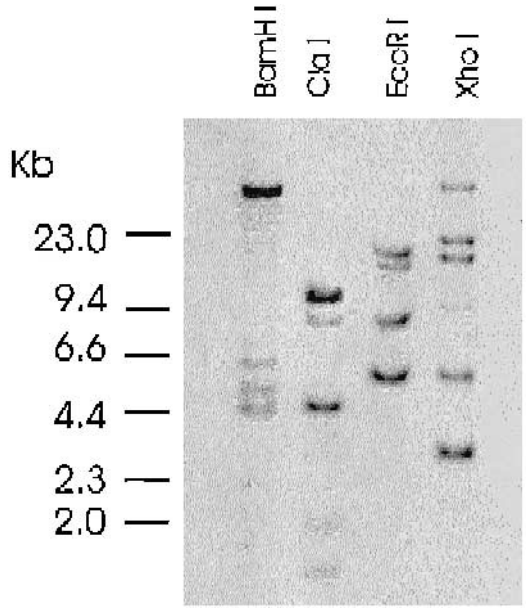

Fig. 2. Southern blot analysis of genomic DNA isolated from *C. sinensis* cv. 'Valencia' young, fully expanded, leaves. Five micrograms of genomic DNA were digested with *Eco*RI, *Bam*HI, and *Xho*I and hybridized with the entire 1079 bp pACVC-1 probe. A 1 kb ladder (Life Technologies) was used as the size standard.

pACVC-1 is homologous to genes for class I and II plant chitinases and shares significant ($>50\%$) sequence similarities to tobacco class I and II enzymes. The polypeptide encoded by the pACVC-1 clone is a class II plant chitinase based on the criteria of Meins et al. [19]. Namely, ACVC-1 lacks the cysteine-rich N-terminal region that categorizes class I chitinases and may have arisen by transposition [23]. However, the encoded polypeptide shares higher sequence similarity with tobacco class I chitinases than with tobacco class II chitinases. ACVC-1 also contains a short, seven C-terminal motif NLMAQSM that is similar to the motif found in class I chitinases, but is not present in the class II chitinases examined. It is not known where ACVC-1 accumulates in the cell. The presence of the short C-terminal motif suggests that ACVC-1 is targeted to the vacuole [24]. The estimated p*I* of 4.52 for ACVC-1 mature polypeptide (aa 43–292) is similar to the p*I* of 4.56 reported for AN4-AN1. If ACVC-1 is targeted to the vacuole it would require an N-terminal targeting sequence to the endoplasmic reticulum [25]. ACVC-1 does contain a putative 42 amino acid, N-terminal, eukaryotic, secretory signal sequence containing a cleavage site (Fig. 1).

pACVC-1 hybridizing mRNA is present in young green bark of 'Valencia' sweet orange seedlings, but was not detected in juvenile or mature leaves, roots, or flavedo. Attempts to obtain sufficient mRNA samples from mature bark were unsuccessful, so we could not determine if expression in bark is limited to specific developmental stages. Developmentally-dependent expression of some chitinases is known in plants [26–28]. In addition, we infested 'Valencia' seedlings with *Diaprepes abbreviatus* larvae as we have done previously for citrus rootstock varieties and analyzed the root tissues for chitinases by enzyme assay and immunodetection [11]. Larval herbivory of some rootstocks (e.g., sour orange) induced chitinases; however, we were unable to detect any induction either by enzyme assay, immunodetection, or pACVC-1 mRNA hybridization (data not shown).

It is unknown if ACVC-1 has chitinase activity, and if it does, if it has any role in a defensive response of the plant to pathogen invasion. Chitinases have been reported that are constitutively expressed and developmentally regulated [27,29] and may play no role in disease resistance. De Jong et al. [26] reported that a glycosylated acidic endochitinase was

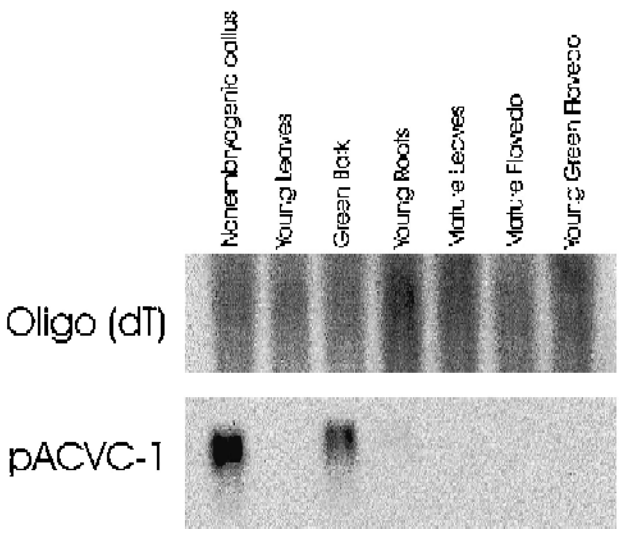

Fig. 3. Northern blot analysis of pACVC-1 transcripts in 'Valencia' sweet orange callus cultures (Val88-1) and in 'Valencia' sweet orange tissues. Oligo(dT) 30-mer probe used to verify the presence and transfer of mRNA onto the hybridization membrane. 2 Tg mRNA/lane.

essential in permitting somatic embryos of carrot to develop beyond the globular stage. Cellular localization studies and fungal growth inhibition assays will be necessary to clarify ACVC-1's role in the plant.

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